USE OF NITRITE SALTS FOR THE TREATMENT OF CARDIOVASCULAR CONDITIONS

Cross Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/485,959, filed July 9, 2003, and No. 60/511,244, filed October 14, 2003, both of which are incorporated herein by reference in their entirety.

Background of the Disclosure

The last decade has seen an increase in the understanding of the critical role nitric oxide as a blood vessel dilator contributing to the regulation of blood flow and cardiovascular homeostasis. Nitric oxide may be oxidized in blood to nitrite (NO_2-), an anion considered to be an inert metabolic end product of such nitric oxide oxidation. *In vivo* plasma levels of nitrite have been reported to range from 150 to 1000 nM, and the nitrite concentration in aortic ring tissue has been reported to be in excess of 10,000 nM (Rodriguez *et al.*, *Proc Natl Acad Sci U S A, 100*, 336-41, 2003; Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; and Rassaf *et al.*, *Nat Med, 9*, 481-3, 2003). This potential storage pool for NO is in excess of plasma S-nitrosothiols, which have been reported to be less than 10 nM in human plasma (Rassaf *et al.*, *Nat Med, 9*, 481-3, 2003; Rassaf *et al.*, *Free Radic Biol Med, 33*, 1590-6, 2002; Rassaf *et al.*, *J Clin Invest, 109*, 1241-8, 2002; and Schechter *et al.*, *J Clin Invest, 109*, 1149-51, 2002). Mechanisms have been proposed for the *in vivo* conversion of nitrite to NO, for example, by enzymatic reduction by xanthine oxidoreductase or by non-enzymatic disproportionation/acidic reduction (Millar *et al.*, *Biochem Soc Trans, 25*, 528S, 1997; Millar *et al.*, *FEBS Lett, 427*, 225-8, 1998; Godber *et al.*, *J Biol Chem, 275*, 7757-63, 2000; Zhang *et al.*, *Biochem Biophys Res Commun, 249*, 767-72, 1998 [published erratum appears in *Biochem Biophys Res Commun 251*, 667, 1998]; Li *et al.*, *J Biol Chem, 276*, 24482-9, 2001; Li *et al.*, *Biochemistry, 42*, 1150-9, 2003; Zweier *et al.*, *Nat Med, 1*, 804-9, 1995; Zweier *et al.*, *Biochim Biophys Acta, 1411*, 250-62, 1999; and Samouilov *et al.*, *Arch Biochem Biophys, 357*:1-7, 1998).

Arterial-to-venous gradients of nitrite across the human forearm at rest and during regional NO synthase inhibition have been observed, with increased consumption of nitrite occurring with exercise (Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; Gladwin *et al.*, *Proc Natl Acad Sci USA, 97*, 11482-11487, 2000; and Cicinelli *et al.*, *Clin Physiol, 19*:440-2, 1999). Kelm and colleagues have reported that large artery-to-vein gradients of nitrite form across the human forearm during NO synthase inhibition (Lauer *et al.*, *Proc Natl Acad Sci USA, 98*, 12814-9, 2001). Unlike the more simple case of oxygen extraction across a vascular bed, nitrite may be both consumed, as evidenced by artery-to-vein gradients during NO synthase inhibition and exercise, and produced in the vascular bed by endothelial nitric oxide synthase-derived NO reactions with oxygen.

At high concentrations, nitrite has been reported to be a vasodilator *in vitro* (Ignarro *et al.*, *Biochim Biophys Acta, 631*, 221-31, 1980; Ignarro *et al.*, *J Pharmacol Exp Ther, 218*, 739-49, 1981; Moulds *et al.*, *Br J Clin Pharmacol, 11*, 57-61, 1981; Gruetter *et al.*, *J Pharmacol Exp Ther, 219*,

181-6, 1981; Matsunaga *et al.*, *J Pharmacol Exp Ther, 248*, 687-95, 1989; and Laustiola *et al.*, *Pharmacol Toxicol, 68*, 60-3, 1991). The levels of nitrite shown to vasodilate *in vitro* have always been in excess of 100,000 nM (100 μM) and usually at millimolar concentrations.

Consistent with the high concentrations of nitrite required to vasodilate *in vitro*, when Lauer and colleagues infused nitrite into the forearm circulation of human subjects, they reported no vasodilatory effects, even with concentrations of 200 μM in the forearm (Lauer *et al.*, *Proc Natl Acad Sci USA, 98*, 12814-9, 2001). Lauer *et al.* reported that a "complete lack of vasodilator activity of intraartierial infusions of nitrite clearly rules out any role for this metabolite in NO delivery" and concluded that "physiological levels of nitrite are vasodilator-inactive." Furthermore, Rassaf and colleagues also failed to find a vasodilatory effect in humans following infusion of nitrite (Rassaf *et al.*, *J Clin Invest, 109*, 1241-8, 2002). Thus, *in vivo* studies have concluded that physiological levels of nitrites do not serve as a source for NO, and that physiological levels of nitrites do not have a role in regulating blood pressure.

Historically, nitrite has been used as a treatment for cyanide poisoning. High concentrations are infused into a subject suffering cyanide poisoning in order to oxidize hemoglobin to methemoglobin, which will bind cyanide. These high concentrations of nitrite produce clinically significant methemoglobinemia, potentially decreasing oxygen delivery. While these high concentrations of nitrite have been shown to decrease blood pressure in humans,. the amount of methemoglobin formed precluded a use for nitrite in the treatment of other medical conditions.

Therefore, the state of the art was that nitrite was not a significant vasodilator at concentrations below 100 μM *in vitro*, and even when infused into humans at concentrations of 200 μM in the forearm. It was also the state of the art that nitrite was not converted to nitric oxide in the human blood stream.

Summary of the Disclosure

It has been surprisingly discovered that administration of pharmaceutically-acceptable salts of nitrite is useful in the regulation of the cardiovascular system. It has also been surprisingly discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. These effects surprisingly occur at doses that do not produce clinically significant methemoglobinemia. These discoveries now enable methods to prevent and treat conditions associated with the cardiovascular system, for example, high blood pressure, pulmonary hypertension, cerebral vasospasm and tissue ischemia-reperfusion injury. These discoveries also provide methods to increase blood flow to tissues, for example, to tissues in regions of low oxygen tension. It is particularly surprising that the nitrite does not need to be applied in an acidified condition in order for it to be effective in regulating the cardiovascular system, and more particularly to act as a vasodilator *in vivo*.

It has been demonstrated by the inventors that nitrite can serve as a vasodilator in humans at much lower concentrations (as low as 0.9 μM) than have been used in the past for cyanide poisoning.

The mechanism is believed to involve a reaction of nitrite with deoxygenated hemoglobin and red blood cells, to produce the vasodilating gas nitric oxide. This potent biological effect is observed at doses of nitrite that do not produce clinically significant methemoglobininemia (for instance, less than 20%, more preferably less than 5% methemoglobin in the subject).

It has been discovered that nitrite is converted to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. Further, it has been surprisingly discovered that administration of nitrite, for instance a pharmaceutically-acceptable salt of nitrite, to a subject causes a reduction in blood pressure and an increase in blood flow to tissues, for example, to tissues in regions of low oxygen tension. These discoveries now enable useful methods to regulate the cardiovascular system, for instance to prevent and treat malconditions associated with the cardiovascular system, for example, high blood pressure, or organs, tissues, or systems suffering a lack of or inadequate blood flow. Non-limiting examples of contemplated malconditions include stroke, heart disease, kidney disease and failure, eye damage including hypertensive retinopathy, diabetes, and migraines.

In one example embodiment, the present disclosure provides a method for decreasing a subject's blood pressure or increasing blood flow, including in a particular embodiment administering to the subject sodium nitrite at about 36 μmoles per minute into the forearm brachial artery.

The present disclosure additionally provides a method for increasing blood flow to a tissue of a subject, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite, such as a salt thereof, so as to increase blood flow to a tissue of the subject. The blood flow may be specifically increased in tissues in regions of low oxygen tension. The present disclosure also provides a method for decreasing a subject's blood pressure, comprising administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to decrease the subject's blood pressure.

The present disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure or reduced blood flow, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure.

Also provided is a method for treating a subject having a hemolytic condition, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

The disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure in the lungs, *e.g.* pulmonary hypertension, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite. In some embodiments, this includes treating a subject having neonatal pulmonary hypertension. In some embodiments, this includes treating a subject having primary and/or secondary pulmonary hypertension. In some embodiments for treating subject s having a condition associated with elevated blood pressure in the lungs, the nitrite is nebulized.

Also contemplated herein are methods for treating, ameliorating, or preventing other conditions of or associated with blood flow, including vasospasm, stroke, angina, revascularization of coronary arteries and other arteries (peripheral vascular disease), transplantation (*e.g.*, of kidney, heart, lung, or liver), treatment of low blood pressure (such as that seen in shock or trauma, surgery and cardiopulmonary arrest) to prevent reperfusion injury to vital organs, cutaneous ulcers (*e.g.*, with topical, non-acidified nitrite salt), Raynauds phenomenon, and treatment of hemolytic conditions (such as sickle cell, malaria, TTP, and HUS) and other conditions listed herein.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

Brief Description of the Figures

Figure 1 is a graph, depicting hemodynamic and metabolic measurements at baseline and during exercise in 18 subjects. **Figure 1A** shows effects on each of the indicated values without inhibition of NO synthesis. **Figure 1B** shows effects with inhibition of NO synthesis. *Key*: MAP – mean arterial pressure, mmHg; FBF – forearm blood flow, mL/min/100mL; O_2 saturation, %; pO_2 – venous oxyhemoglobin saturation, partial pressure of oxygen, mmHg; pH, units; * = $p<0.05$ vs. Baseline 1 or 2, respectively; ** = $p<0.01$ vs. Baseline 1 or 2, respectively; † = $p<0.05$ vs. Baseline 1; †† = $p<0.01$ vs. Initial Exercise.

Figure 2 is a graph, depicting effects of infusion of sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 18 healthy subjects. **Figure 2A** shows effects on each of the indicated values without inhibition of NO synthesis. **Figure 2B** shows effects with inhibition of NO synthesis. *Key as for Figure 1, plus*: Nitrite – venous nitrite, μM; NO-heme – venous iron-nitrosyl-hemoglobin, μM; and MetHb – venous methemoglobin, %; + = $p<0.01$ vs. Initial Exercise.

Figure 3 is a series of graphs, illustrating the effects of infusion of low-dose sodium nitrite into the brachial arteries of 10 healthy subjects at baseline and during exercise, without and with inhibition of NO synthesis. **Figure 3A** shows forearm blood flow at baseline and following a five-minute infusion of $NaNO_2$. **Figure 3B** shows forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion with and without exercise stress. **Figure 3C** shows venous levels of nitrite from the forearm circulation at the time of blood flow measurements. **Figure 3D** shows venous levels of S-nitroso-hemoglobin (S-NO) and iron-nitrosyl-hemoglobin (Hb-NO) at baseline and following nitrite infusion during exercise stress.

Figure 4 is a pair of graphs, showing formation of NO-hemoglobin adducts. **Figure 4A** shows formation of iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin, comparing baseline, with nitrite infusion, and nitrite infusion with exercise. **Figure 4B** compares formation of NO-hemoglobin adducts with hemoglobin-oxygen saturation in the human circulation, during nitrite infusion.

Figure 5A shows NO release following nitrite injections into solutions of PBS ("PBS"), deoxygenated red blood cells ("deoxy-RBC"), and oxygenated red blood cells ("oxy-RBC"). **Figure**

5B shows the rate of NO formation from nitrite mixed with PBS (first bar in each set), and oxygenated and deoxygenated red blood cells (second and third bar in each set, respectively).

Detailed Description of the Disclosure

I. Abbreviations

ANOVA	analysis of variance
deoxy-RBC	deoxygenated red blood cells
FBF	forearm blood flow
L-NMMA	L-NG-monomethyl-arginine
NO	nitric oxide
NOS	nitric oxide synthase
MAP	mean arterial pressure
MetHb	methemoglobin
oxy-RBC	oxygenated red blood cells
PBS	phosphate buffered saline
pO_2 (or Po_2)	partial oxygen pressure
S-NO	S-nitroso-hemoglobin

II. Terms

Unless otherwise noted, terms used herein should be accorded their standard definitions and conventional usage. For example, one of skill in the art can obtain definitions for the terms used herein in dictionaries and reference textbooks, for example: *Stedman's Medical Dictionary* (26th Ed., Williams and Wilkins, Editor M. Spraycar, 1995); *The New Oxford American Dictionary* (Oxford University Press, Eds E. Jewell and F. Abate, 2001); *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 3rd Ed., Cold Spring Harbor Laboratory Press, 2001); and *Hawley's Condensed Chemical Dictionary*, 11th Ed. (Eds. N. I. Sax and R. J. Lewis, Sr., Van Nostrand Reinhold, New York, New York, 1987); *Molecular Biology and Biotechnology: a Comprehensive Desk Reference* (VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8)).

In order to facilitate review of the various embodiments, the following explanations of specific terms are provided:

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals.

Cerebral ischemia or ischemic stroke: A condition that occurs when an artery to or in the brain is partially or completely blocked such that the oxygen demand of the tissue exceeds the oxygen supplied. Deprived of oxygen and other nutrients following an ischemic stroke, the brain suffers damage as a result of the stroke.

Ischemic stroke can be caused by several different kinds of diseases. The most common problem is narrowing of the arteries in the neck or head. This is most often caused by atherosclerosis, or gradual cholesterol deposition. If the arteries become too narrow, blood cells may collect in them and form blood clots (thrombi). These blood clots can block the artery where they are formed (thrombosis), or can dislodge and become trapped in arteries closer to the brain (embolism).

Another cause of stroke is blood clots in the heart, which can occur as a result of irregular heartbeat (for example, atrial fibrillation), heart attack, or abnormalities of the heart valves. While these are the most common causes of ischemic stroke, there are many other possible causes. Examples include use of street drugs, traumatic injury to the blood vessels of the neck, or disorders of blood clotting.

Ischemic stroke is by far the most common kind of stroke, accounting for about 80% of all strokes. Stroke can affect people of all ages, including children. Many people with ischemic strokes are older (60 or more years old), and the risk of stroke increases with older ages. At each age, stroke is more common in men than women, and it is more common among African-Americans than white Americans. Many people with stroke have other problems or conditions which put them at higher risk for stroke, such as high blood pressure (hypertension), heart disease, smoking, or diabetes.

Hypoxia: Deficiency in the amount of oxygen reaching body tissues.

Injectable composition: A pharmaceutically acceptable fluid composition comprising at least one active ingredient, for example, a salt of nitrite. The active ingredient is usually dissolved or suspended in a physiologically acceptable carrier, and the composition can additionally comprise minor amounts of one or more non-toxic auxiliary substances, such as emulsifying agents, preservatives, pH buffering agents and the like. Such injectable compositions that are useful for use with the compositions of this disclosure are conventional; appropriate formulations are well known in the art.

Ischemia: A vascular phenomenon in which a decrease in the blood supply to a bodily organ, tissue, or part is caused, for instance, by constriction or obstruction of one or more blood vessels. Ischemia sometimes results from vasoconstriction or thrombosis or embolism. Ischemia can lead to direct ischemic injury, tissue damage due to cell death caused by reduced oxygen supply.

Ischemia/reperfusion injury: In addition to the immediate injury that occurs during deprivation of blood flow, ischemic/reperfusion injury involves tissue injury that occurs after blood flow is restored. Current understanding is that much of this injury is caused by chemical products and free radicals released into the ischemic tissues.

When a tissue is subjected to ischemia, a sequence of chemical events is initiated that may ultimately lead to cellular dysfunction and necrosis. If ischemia is ended by the restoration of blood flow, a second series of injurious events ensue producing additional injury. Thus, whenever there is a transient decrease or interruption of blood flow in a subject, the resultant injury involves two components - the direct injury occurring during the ischemic interval and the indirect or reperfusion injury that follows. When there is a long duration of ischemia, the direct ischemic damage, resulting from hypoxia, is predominant. For relatively short duration ischemia, the indirect or reperfusion mediated damage becomes increasingly important. In some instances, the injury produced by reperfusion can be more severe than the injury induced by ischemia *per se*. This pattern of relative contribution of injury from direct and indirect mechanisms has been shown to occur in all organs.

Methemoglobin: The oxidized form of hemoglobin in which the iron in the heme component has been oxidized from the ferrous (+2) to the ferric (+3) state. This renders the hemoglobin molecule incapable of effectively transporting and releasing oxygen to the tissues. Normally, there is about 1% of total hemoglobin in the methemoglobin form.

Methemoglobinemia: A condition in which a substantial portion of the hemoglobin in the blood of a subject is in the form of methemoglobin, making it unable to carry oxygen effectively to the tissues. Methemoglobinemia can be an inherited disorder, but it also can be acquired through exposure to chemicals such as nitrates (nitrate-contaminated water), aniline dyes, and potassium chlorate. It is not the presence of methemoglobin but the amount that is important in the clinical setting. The following provides rough indications of symptoms associated with different levels of methemoglobin in the blood: < 1.7%, normal; 10-20%, mild cyanosis (substantially asymptomatic, though it can result in "chocolate brown" blood); 30-40%, headache, fatigue, tachycardia, weakness, dizziness; >35%, symptoms of hypoxia, such as dyspnea and lethargy; 50-60%, acidosis, arrhythmias, coma, convulsions, bradycardia, severe hypoxia, seizures; >70% usually results in death.

Nitrite: The inorganic anion $^{-}NO_2$ or a salt of nitrous acid (NO_2^{-}). Nitrites are often highly soluble, and can be oxidized to form nitrates or reduced to form nitric oxide or ammonia. Nitrite may form salts with alkali metals, such as sodium ($NaNO_2$, also known as nitrous acid sodium salt), potassium and lithium, with alkali earth metals, such as calcium, magnesium and barium, with organic bases, such as amine bases, for example, dicyclohexylamine, pyridine, arginine, lysine and the like. Other nitrite salts may be formed from a variety of organic and inorganic bases. In particular embodiments, the nitrite is a salt of an anionic nitrite delivered with a cation, which cation is selected from sodium, potassium, and arginine. Many nitrite salts are commercially available, and/or readily produced using conventional techniques.

Parenteral: Administered outside of the intestine, for example, not via the alimentary tract. Generally, parenteral formulations are those that will be administered through any possible mode except ingestion. This term especially refers to injections, whether administered intravenously, intrathecally, intramuscularly, intraperitoneally, or subcutaneously, and various surface applications including intranasal, intradermal, and topical application, for instance.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the compounds herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to

biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Peripheral Vascular Disease (PVD): A condition in which the arteries that carry blood to the arms or legs become narrowed or occluded. This interferes with the normal flow of blood, sometimes causing pain but often causing no readily detectable symptoms at all.

The most common cause of PVD is atherosclerosis, a gradual process in which cholesterol and scar tissue build up, forming plaques that occlude the blood vessels. In some cases, PVD may be caused by blood clots that lodge in the arteries and restrict blood flow. PVD affects about one in 20 people over the age of 50, or 8 million people in the United States. More than half the people with PVD experience leg pain, numbness or other symptoms, but many people dismiss these signs as "a normal part of aging" and do not seek medical help. The most common symptom of PVD is painful cramping in the leg or hip, particularly when walking. This symptom, also known as "claudication," occurs when there is not enough blood flowing to the leg muscles during exercise, such that ischemia occurs. The pain typically goes away when the muscles are rested.

Other symptoms may include numbness, tingling or weakness in the leg. In severe cases, people with PVD may experience a burning or aching pain in an extremity such as the foot or toes while resting, or may develop a sore on the leg or foot that does not heal. People with PVD also may experience a cooling or color change in the skin of the legs or feet, or loss of hair on the legs. In extreme cases, untreated PVD can lead to gangrene, a serious condition that may require amputation of a leg, foot or toes. People with PVD are also at higher risk for heart disease and stroke.

A **"pharmaceutical agent"** or **"drug"** refers to a chemical compound or other composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

Preventing or treating a disease: "Preventing" a disease refers to inhibiting the full development of a disease. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified nitrite salt preparation is one in which the specified nitrite salt is more enriched than it is in its generative environment, for instance within a biochemical reaction chamber. Preferably, a preparation of a specified nitrite salt is purified such that the salt represents at least 50% of the total nitrite content of the preparation. In some embodiments, a purified preparation contains at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% or more of the specified compound, such as a particular nitrite salt.

Reperfusion: Restoration of blood supply to tissue that is ischemic, due to decrease in blood supply. Reperfusion is a procedure for treating infarction or other ischemia, by enabling viable ischemic tissue to recover, thus limiting further necrosis. However, it is thought that reperfusion can itself further damage the ischemic tissue, causing reperfusion injury.

Subject: Living multi-cellular organisms, including vertebrate organisms, a category that includes both human and non-human mammals.

Therapeutic: A generic term that includes both diagnosis and treatment.

Therapeutically effective amount of [a vasodilator]: A quantity of compound, such as a nitrite salt, sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to treat or ameliorate relatively high blood pressure, or to measurably decrease blood pressure over a period of time, or to measurably inhibit an increase in blood pressure, in a subject.

An effective amount of a vasodilator may be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount will be dependent on the compound applied, the subject being treated, the severity and type of the affliction, and the manner of administration of the compound. For example, a therapeutically effective amount of an active ingredient can be measured as the concentration (moles per liter or molar-M) of the active ingredient (such as a pharmaceutically-acceptable salt of nitrite) in blood (*in vivo*) or a buffer (*in vitro*) that produces an effect.

By way of example, as described herein it is now shown that pharmaceutically-acceptable salts of nitrite (such as sodium nitrite) are effective as vasodilators at calculated dosages of about 0.6 to about 200 μM final concentration of nitrite in the circulating blood of a subject, which level can be determined empirically or through calculations. Specific levels can be reached, for instance, by providing less than about 200 mg or less nitrite in a single dose, or a dose provided over a period of time (*e.g.*, by infusion or inhalation). For instance, other dosages may be 150 mg, 100 mg, 75 mg, 50 mg or less. Specific example dosages of nitrite salts are provided herein, though the examples are not intended to be limiting. Exact dosage amounts will vary by the size of the subject being treated, the duration of the treatment, the mode of administration, and so forth.

Particularly beneficial therapeutically effective amounts of a vasodilator, such as a pharmaceutically-acceptable nitrite salt (*e.g.*, sodium nitrite), are those that are effective for vasodilation or increasing blood flow, but not so high that a significant or toxic level of methemoglobin is produced in the subject to which the vasodilator is administered. In specific embodiments, for instance, no more than about 25% methemoglobin is produced in the subject. More preferably, no more than 20%, no more than 15%, no more than 10%, no more than 8% or less methemoglobin is produced, for instance as little as 5% or 3% or less, in response to treatment with the vasodilator.

The compounds discussed herein have equal application in medical and veterinary settings. Therefore, the general term "subject being treated" is understood to include all animals (for example, humans, apes, laboratory animals, companion animals, etc.) that are or may be suffering from an aberration in blood pressure, such as hypertension.

Vasoconstriction. The diminution of the caliber or cross-sectional area of a blood vessel, for instance constriction of arterioles leading to decreased blood flow to a body part. This can be

caused by a specific **vasoconstrictor**, an agent (for instance a chemical or biochemical compound) that causes, directly or indirectly, constriction of blood vessels. Such an agent can also be referred to as a **vasohypertonic** agent, and is said to have **vasoconstrictive** activity. A representative category of vasoconstrictors is the **vasopressor** (from the term pressor, tending to increase blood pressure), which term is generally used to refer to an agent that stimulates contraction of the muscular tissue of the capillaries and arteries.

Vasoconstriction also can be due to vasospasm, inadequate vasodilatation, thickening of the vessel wall, or the accumulation of flow-restricting materials on the internal wall surfaces or within the wall itself. Vasoconstriction is a major presumptive or proven factor in aging and in various clinical conditions including progressive generalized atherogenesis, myocardial infarction, stroke, hypertension, glaucoma, macular degeneration, migraine, hypertension and diabetes mellitus, among others.

Vasodilation. A state of increased caliber of the blood vessels, or the act of dilation of a blood vessel, for instance dilation of arterioles leading to increased blood flow to a body part. This can be caused by a specific **vasodilator**, an agent (for instance, a chemical or biochemical compound) that causes, directly or indirectly, dilation of blood vessels. Such an agent can also be referred to as a **vasohypotonic** agent, and is said to have **vasodilative** activity.

Vasospasm: Another cause of stroke occurs secondary to spasm of blood vessels supplying the brain. This type of stroke typically follows a subarchnoid aneurismal hemorrhage with a delayed development of vasospasm within 2-3 weeks of the bleeding event. A similar type of stroke may complicate sickle cell disease.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Hence "comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Overview of Several Embodiments

It has been surprisingly discovered that administration of pharmaceutically-acceptable salts of nitrite is useful in the regulation of the cardiovascular system. It has also been surprisingly discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. These effects surprisingly occur at doses that do not produce clinically significant methemoglobinemia. These discoveries now enable methods to prevent and treat conditions associated with the cardiovascular system, for example, high blood pressure, pulmonary hypertension, cerebral vasospasm and tissue ischemia-reperfusion injury. These discoveries also provide methods to increase blood flow to tissues, for example, to tissues in regions of low oxygen tension. It is particularly surprising that the nitrite does not need to be applied in an acidified condition in order for it to be effective in regulating the cardiovascular system, and more particularly to act as a vasodilator *in vivo*.

Accordingly, the present disclosure provides in one embodiment a method for decreasing a subject's blood pressure, including administering to the subject sodium nitrite at about 36 μmoles per minute or less into the forearm brachial artery or intravenously.

The present disclosure also provides a method for decreasing a subject's blood pressure, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to decrease (or lower, or reduce) the subject's blood pressure. Another embodiment is a method for treating a subject having a condition associated with elevated blood pressure, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure. Also provided is a method for treating a subject having a hemolytic condition, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

The present disclosure additionally provides a method for increasing blood flow to a tissue of a subject, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to increase blood flow to a tissue of the subject. Also provided is a method for producing an amount of NO in a subject effective the decrease the subject's blood pressure, including administering a pharmaceutically-acceptable nitrite to the subject.

The present disclosure further provides a pharmaceutical composition comprising an effective amount of a pharmaceutically-acceptable nitrite and a carrier.

In some embodiments, the vascular complication is one or more selected from the group consisting of pulmonary hypertension (including neonatal pulmonary hypertension, primary pulmonary hypertension, and secondary pulmonary hypertension), systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, an ischemic central nervous system event, and death.

In some embodiments, nitrite is administered to neonates to treat pulmonary hypertension.

In some embodiments, the hemolytic condition includes one or more selected from: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, cardiopulomonary bypass, and hemodialysis.

In some embodiments, the decreased blood flow to the tissue is caused directly or indirectly by at least one of the following conditions: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, hemodialysis, pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, and an ischemic central nervous system event.

In some embodiments, the tissue is an ischemic tissue. In some embodiments, the administration is parenteral, oral, bucal, rectal, *ex vivo*, or intraocular. In some embodiments, the administration is peritoneal, intravenous, intraarterial, subcutaneous, inhaled, or intramuscular. In some embodiments, the nitrite is administered to the subject in an environment of low oxygen tension, or acts in an area of the subject's body that displays relatively low oxygen tension. In some embodiments, the nitrite is administered as a pharmaceutically-acceptable salt of nitrite, such as, for instance, sodium nitrite, potassium nitrite, or arginine nitrite. In some embodiments, the nitrite is administered in combination with at least one additional active agent. It is specifically contemplated that, in certain embodiments, that the subject is a mammal, for instance, a human.

The disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure in the lungs, *e.g.* pulmonary hypertension, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite. In some embodiments, this

includes treating a subject having neonatal pulmonary hypertension. In some embodiments, this includes treating a subject having primary and/or secondary pulmonary hypertension. In some embodiments for treating subjects having a condition associated with elevated blood pressure in the lungs, the nitrite is nebulized.

Thus, there is provided herein a method for inducing vasodilation and/or increasing blood flow in a subject, which method involves administering to the subject an effective amount of a pharmaceutically-acceptable salt of nitrite for a sufficient period of time to induce vasodilation and/or increase blood flow in the subject. Non-limiting examples of pharmaceutically acceptable salts of nitrite include sodium nitrite, potassium nitrite, and arginine nitrite. In examples of the provided methods, the pharmaceutically-acceptable salt of nitrite reacts in the presence of hemoglobin in the subject to release nitric oxide.

It is a specific advantage of methods provided herein that the effective amount of the pharmaceutically-acceptable salt of nitrite administered to the subject does not induce toxic levels of methemoglobin, and in many embodiments does not induced formation of clinically significant amounts of methemoglobin in the subject. Therefore, contemplated herein are methods in which the effective amount of the pharmaceutically-acceptable salt of nitrite, when administered to the subject, induces production in the subject of no more than about 25% methemoglobin; no more than about 20% methemoglobin; no more than about 10% methemoglobin; no more than about 8% methemoglobin; or no more than about 5% methemoglobin. Beneficially, examples of the provided methods induce production of even less than 5% methemoglobin, for instance no more than about 3% methemoglobin, less than 3%, less than 2%, or even less than 1%.

In one specific example of a method for inducing vasodilation and/or increasing blood flow in a subject, sodium nitrite is administered by injection at about 36 μmoles per minute for at least five minutes into the forearm brachial artery of the subject.

The effective amount of the pharmaceutically-acceptable salt of nitrite is administered, in various embodiments, to a circulating concentration in the subject of about 0.6 to 240 μM, measured locally to the site of administration or generally in the subject. It is noted that the local level of nitrite is expected to be higher than the general circulating level particularly in short delivery regimens; in long term delivery regimens, such as delivery using a pump or injector, or by inhalation, the system-wide or general nitrite level is expected to near the level measured near the administration site.

Administration of the pharmaceutically-acceptable nitrite can be, for instance, parenteral, oral, bucal, rectal, *ex vivo*, or intraocular in certain embodiments. In various embodiments, it is also contemplated that the administration of the nitrite can be peritoneal, intravenous, intraarterial, subcutaneous, inhaled, intramuscular, or into a cardiopulmonary bypass circuit. Combinations of two or more routes of administration are also contemplated.

In various embodiments of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject is a mammal. It is particularly contemplated that the subject can be a human.

Combination therapy methods are contemplated, wherein the nitrite is administered in combination with at least one additional agent. By way of non-limiting examples, the additional agent is one or more selected from the list consisting of penicillin, hydroxyurea, butyrate, clotrimazole, arginine, or a phosphodiesterase inhibitor (such as sildenafil).

In another embodiment of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject has elevated blood pressure, and the method is a method for treating at least one vascular complication associated with the elevated blood pressure, or the subject has a hemolytic condition, and the method is a method for treating at least one vascular complication associated with the hemolytic condition. Optionally, the subject may have both elevated blood pressure and a hemolytic condition.

In examples of the methods provided herein, the at least one vascular complication is one or more selected from the group consisting of pulmonary hypertension, systemic hypertension, peripheral vascular disease, trauma, cardiac arrest, general surgery, organ transplantation, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, angina, an ischemia-reperfusion event, an ischemic central nervous system event, and death.

In examples of the methods in which the subject has a hemolytic condition, the hemolytic condition is one or more selected from the group consisting of sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, and hemodialysis.

In yet another embodiment of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject has a condition associated with decreased blood flow to a tissue, and the method is a method to increase blood flow to the tissue of the subject. For instance, in examples of this method, the decreased blood flow to the tissue is caused directly or indirectly by at least one condition selected from the group consisting of: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic

autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, hemodialysis, pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, and an ischemic central nervous system event.

It is specifically contemplated in examples of this method that the tissue is an ischemic tissue, for instance one or more tissues selected from the group consisting of neuronal tissue, bowel tissue, intestinal tissue, limb tissue, lung tissue, central nervous tissue, or cardiac tissue.

Also provided are methods for inducing vasodilation and/or increasing blood flow in a subject having elevated blood pressure, wherein the elevated blood pressure comprises elevated blood pressure in the lungs. By way of example, it is contemplated that such subject in some instances has neonatal pulmonary hypertension, or primary and/or secondary pulmonary hypertension.

In examples of embodiments where the elevated blood pressure, or need for increased blood flow, in the subject comprises elevated blood pressure or need for increased blood flow in the lungs, the pharmaceutically-acceptable salt of nitrite is nebulized.

By way of example, in various embodiments the pharmaceutically-acceptable salt of nitrite is administered to a circulating concentration in the subject of no more than about 100 μM; no more than about 50 μM; no more than about 20 μM; no more than about 16 μM; or less than about 16 μM.

IV. Sodium Nitrite as an in vivo *vasodilator*

Nitrite anions are present in concentrations of about 150-1000 nM in the plasma and about 10 μM in aortic tissue. This represents the largest vascular storage pool of nitric oxide (NO), provided physiological mechanisms exist to reduce nitrite to NO. The vasodilator properties of nitrite in the human forearm and the mechanisms extant for its bioactivation have been investigated and results are reported herein. Sodium nitrite was infused at about 36 μmoles per minute into the forearm brachial artery of 18 normal volunteers, resulting in a regional nitrite concentration of about 222 μM and an immediate about 175% increase in resting forearm blood flow. Increased blood flow was observed at rest, during NO synthase inhibition and with exercise, and resulted in increased tissue perfusion, as demonstrated by increases in venous hemoglobin-oxygen saturation, partial pressure of oxygen, and pH. Systemic concentrations of nitrite increased to about 16 μM and significantly reduced mean arterial blood pressure. In an additional six subjects, the dose of nitrite was reduced about 2-logs and infused at 360 nmoles per minute, resulting in a forearm nitrite concentration of about 2 μM and an about 22% increase in blood flow.

Nitrite infusions were associated with the formation of erythrocyte iron-nitrosyl-hemoglobin, and to a lesser extent, S-nitroso-hemoglobin across the forearm vasculature. The formation of NO-modified hemoglobin appears to result from the nitrite reductase activity of deoxyhemoglobin, linking tissue hypoxia and nitrite bioactivation.

These results indicate that physiological levels of blood and tissue nitrite represent a major bioavailable pool of NO that contributes to vaso-regulation and provides a mechanism for hypoxic vasodilation via reaction of vascular nitrite with deoxygenated heme proteins. Substantial blood flow effects of nitrite infusion into the brachial artery of normal human subjects results from forearm nitrite concentrations as low as about 0.9μM.

By way of example, as described herein it is now shown that pharmaceutically-acceptable salts of nitrite (such as sodium nitrite) are effective as vasodilators at calculated dosages of about 0.6 to about 200 μM final concentration of nitrite in the circulating blood of a subject. Specific circulating levels (locally or generally in the subject) can be reached, for instance, by providing less than about 200 mg or less nitrite in a single dose, or a dose provided over a period of time (*e.g.*, by infusion or inhalation). For instance, other dosages may be 150 mg, 100 mg, 75 mg, 50 mg or less. Specific example dosages of nitrite salts are provided herein, though the examples are not intended to be limiting. Exact dosage amounts will vary by the size of the subject being treated, the duration of the treatment, the mode of administration, and so forth.

Infusion rates can be calculated, for any given desired target circulating concentration, by using the following equation:

Infusion rate (μM/min) = target concentration (μmol/L, or μM) x Clearance (L/min)
where Clearance (L/min) = 0.015922087 x weight of the subject (kg) ↑0.8354

The rate of clearance has been calculated based on empirical results, including those reported herein.

For instance, when sodium nitrite is infused into a human forearm at 36 micromoles (μMol) per minute, the concentration measured coming out of forearm is about 222 μM and about 16 μM in whole body, after 15 minutes infusion. The background level of circulating nitrite in mammals is low, around 150-500 nanoM.

Particularly beneficial therapeutically effective amounts of a vasodilator, such as a pharmaceutically-acceptable nitrite salt (*e.g.*, sodium nitrite), are those that are effective for vasodilation or increasing blood flow, but not so high that a significant or toxic level of methemoglobin is produced in the subject to which the vasodilator is administered. In specific embodiments, for instance, no more than about 25% methemoglobin is produced in the subject. More preferably, no more than 20%, no more than 15%, no more than 10%, no more than 8% or less methemoglobin is produced, for instance as little as 5% or 3% or less, in response to treatment with the vasodilator.

By way of specific example, nitrite can be infused at concentrations less than 40 μMol per minute intravenously or intraarterially, or given by mouth. Importantly, doses used are less than those used for the treatment of cyanide poisoning, which are designed to induce clinically significant methemoglobinemia. Surprisingly, the doses described herein for the treatment/prevention of cardiovascular conditions produce significant and beneficial clinical effects without clinically significant methemoglobin production.

Relatively complex inorganic/organic nitrite compounds and nitrate compounds have been utilized clinically to treat disorders, including angina. These drugs (*e.g,* glyceryl trinitrate) suffer from tolerance (requiring increases in dosage in order to maintain the same effect), however, and are distinct vasodilators compared to nitrite. For example, the former require cellular thiols for metabolism, whereas nitrite or the nitrite salts discussed herein (*e.g.*, sodium nitrite) do not.

V. A mechanism of iron-nitrosyl- and S-nitroso-hemoglobin formation in vivo

The levels of both iron-nitrosyl- and S-nitroso-hemoglobin formed *in vivo* in this study are striking. During a transit time of less than 10 seconds through the forearm circulation during exercise, infused nitrite (200 μM regional concentration) produced approximately 750 nM iron-nitrosyl-hemoglobin and 200 nM SNO-Hb. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation, which fell during exercise stress, measured from the antecubital vein by co-oximetry (for iron-nitrosyl-hemoglobin r=-0.7, P<0.0001; for S-nitroso-hemoglobin r=-0.45, P=0.04; Figure 4B). Addition of 200 μM nitrite to whole blood at different oxygen tensions (0-100%) recapitulated the *in vivo* data with increasing concentrations of iron-nitrosyl hemoglobin being formed at lower oxygen tensions (for iron-nitrosyl-hemoglobin r=-0.968, P<0.0001; for S-nitroso-hemoglobin r=-0.45, P=0.07), strongly suggesting that the NO and SNO formation was dependent on the reaction of nitrite with deoxyhemoglobin.

These data are consistent with the reaction of nitrite with deoxyhemoglobin to form NO and iron-nitrosyl-hemoglobin (Doyle *et al.*, *J Biol Chem, 256*, 12393-12398, 1981). Nitrite is first reduced to form NO and methemoglobin with a rate constant of 2.9 $M^{-1}sec^{-1}$ (measured at 25°C, pH 7.0). This reaction will be pseudo-first order, governed by the amounts (20 mM) of intra-erythrocytic hemoglobin, and limited by the rate of nitrite uptake by the erythrocyte membrane. NO then binds to deoxyhemoglobin to form iron-nitrosyl-hemoglobin, escapes the erythrocyte, or reacts with other higher oxides, such as NO_2, to form N_2O_3 and S-nitroso-hemoglobin.

Equation series 1

NO_2^- (nitrite) + $HbFe^{II}$ (deoxyhemoglobin) + $H^+ \rightarrow$ $HbFe^{III}$ (methemoglobin) + NO + OH^-

NO + $HbFe^{II}$ (deoxyhemoglobin) $\rightarrow$ $HbFe^{II}NO$ (iron-nitrosyl-hemoglobin)

The formation of significant amounts of S-nitroso-hemoglobin *in vivo* during nitrite infusion was also observed. Luschinger and colleagues (*Proc Natl Acad Sci USA, 100*, 461-6, 2003) recently

proposed that nitrite reacts with deoxyhemoglobin to make iron-nitrosyl-hemoglobin, with subsequent "transfer" of the NO to the cysteine 93 to form S-nitroso-hemoglobin mediated by reoxygenation and quaternary T to R transition of hemoglobin. However, a direct transfer of NO from the heme to the thiol requires NO oxidation to NO+ and such "cycling" has not been reproduced by other research groups. Fernandez and colleagues have recently suggested that nitrite catalyzes the reductive nitrosylation of methemoglobin by NO, a process that generates the intermediate nitrosating species dinitrogen teraoxide (N_2O_3) (*Inorg Chem, 42*, 2-4, 2003). However, nitrite reactions with hemoglobin provide ideal conditions for NO and S-nitrosothiol generation along the oxygen gradient as nitrite reacts with deoxyhemoglobin to form NO and with oxyhemoglobin to form nitrogen dioxide (NO_2) radical. NO_2 participates in radical-radical reactions ($k=10^9$ $M^{-1}sec^{-1}$) with NO to form N_2O_3 and S-nitrosothiol. Additional chemistry of nitrite with hemoglobin produces reactive oxygen metabolites (such as superoxide and hydrogen peroxide; Watanabe *et al.*, *Acta Med Okayama 35*, 173-8, 1981; Kosaka *et al.*, *Biochim Biophys Acta 702*, 237-41, 1982; and Kosaka *et al.*, *Environ Health Perspect 73*, 147-51, 1987). Chemistry involving such NO radical- oxygen radical reactions provides competitive pathways for S-nitrosothiol formation in the presence of high affinity NO sinks, such as hemoglobin.

VI. Physiological Considerations

The last decade has seen an increase in the understanding of the critical role nitric oxide (NO) plays in vascular homeostasis. The balance between production of NO and scavenging of NO determines NO bioavailability, and this balance is carefully maintained in normal physiology. The homeostatic, vasoregulatory system is apparently fine-tuned to scavenge excess NO to limit gross endocrine actions while allowing for sufficient local NO necessary for regional tonic vasodilation. However, rapid NO scavenging by cell-free hemoglobin disrupts this balance (Reiter *et al.*, *Nat Med* 8, 1383-1389, 2002). Under normal physiological conditions, hemoglobin is rapidly and effectively cleared by the hemoglobin scavenger system. However, chronic hemolytic conditions, such as sickle cell disease, result in the daily release of substantial quantities of hemoglobin into the vasculature, suggesting that cell-free hemoglobin may have major systemic effects on NO bioavailability. A current focus of research attempts to explain and treat the vascular complications common to many chronic hemolytic conditions, such as pulmonary hypertension, cutaneous ulceration and acute and chronic renal failure. Similarly, a number of clinical diseases and therapies such as acute hemolytic crises, hemolysis during cardiopulmonary bypass procedures, transfusion of aged blood, and myoglobinuria following muscle infarction are often complicated by acute pulmonary and systemic hypertension, acute renal failure, intravascular thrombosis, ischemic central nervous system events and/or death.

It is demonstrated herein that nitrite produces vasodilation in humans associated with nitrite reduction to NO by deoxyhemoglobin. Remarkably, systemic levels of 16 μM resulted in systemic vasodilation and decreased blood pressure, and regional forearm levels of only 1-2 μM significantly

increased blood flow at rest and with exercise stress. Furthermore, conversion of nitrite to NO and S-nitrosothiol was mediated by reaction with deoxyhemoglobin, providing a mechanism for hypoxia-regulated catalytic NO production by the erythrocyte or endothelial/tissue heme proteins. While high concentrations of hemoglobin in red cells, coupled with the near diffusion-limited reaction rates (~10^7 $M^{-1}s^{-1}$) of NO with hemoglobin, seem to prohibit NO from being exported from the red blood cell, the data presented herein argue to the contrary. While not intending to be limiting, perhaps unique characteristics of the erythrocyte membrane, with a submembrane protein and methemoglobin-rich microenvironment, and the relative lipophilic nature of NO, allow compartmentalized NO production at the red blood cell membrane. This, coupled with the small yields of NO necessary for vasodilation, could account for the export of NO despite these kinetic constraints. It is further proposed that *in vivo* chemistry for the conversion of nitrite to NO and S-nitrosothiol by reaction with deoxyhemoglobin and methemoglobin provides a mechanism for hypoxia-regulated catalytic NO production by the erythrocyte or endothelial tissue heme proteins.

Three factors uniquely position nitrite, rather than S-nitrosothiol, as the major vascular storage pool of NO: 1) Nitrite is present in substantial concentrations in plasma, erythrocytes and in tissues (Rodriguez *et al., Proc Natl Acad Sci USA* 100:336-341, 2003). 2) Nitrite is relatively stable, because it is not readily reduced by intracellular reductants, as are S-nitrosothiols (Gladwin *et al., J Biol Chem* 21:21, 2002) and its reaction rate with heme proteins is 10,000 times less than that of authentic NO. 3) Nitrite is only converted to NO by reaction with deoxyhemoglobin (or presumably deoxy-myoglobin, -cytoglobin, and -neuroglobin) and its "leaving group" is the met(ferric)heme protein which will not scavenge and inactivate NO (Doyle *et al., J Biol Chem* 256:12393-12398, 1981). Therefore, this pool provides the ideal substrate for NO generation during hypoxia, providing a novel mechanism for hypoxic vasodilation.

Because a deoxyhemoglobin-nitrite reductase system would result in NO formation in deoxygenating blood, such a system links hemoglobin oxygenation status to NO generation, the principle previously ascribed to S-nitroso-hemoglobin (Jia *et al., Nature* 380:221-226, 1996). Hemoglobin possesses anionic binding cavities that retain nitrite (Gladwin *et al., J Biol Chem* 21:21, 2002) and nitrite is taken up by erythrocytes through the anion exchange protein (AE1 or Band 3) or through the membrane as nitrous acid (a pH dependent process that accelerates nitrite uptake during tissue hypoxia (Shingles *et al., J Bioenerg Biomembr* 29:611-616, 1997; May *et al., Am I Physiol Cell Physiol* 279:C1946-1954, 2000). Such nitrite would provide a steady source of NO, NO_2 and S-nitrosothiol generation that would occur preferentially in hypoxic vascular territories. Because the AE1 protein binds both deoxyhemoglobin and methemoglobin and may channel nitrite, AE1 could serve to localize catalytic NO and S-nitrosothiol generation at the erythrocyte membrane, where the relatively lipophilic NO, NO_2 and N_2O_3 could react in the vicinal lipid bilayer (Figure 5). The erythrocyte membrane is lined by an unstirred outer diffusion barrier and an inner methemoglobin rich protein matrix that might further promote such NO and NO_2 chemistry (Coin *et al., J Biol Chem*

254:1178-1190, 1979; Liu *et al., J Biol Chem* 273:18709-18713, 1998; Han *et al., Proc Natl Acad Sci USA* 99:7763-7768, 2002).

This model is consistent with the *in vitro* observations of Pawloski and colleagues (Pawloski *et al., Nature* 409:622-626, 2001) showing that S-nitrosation of hemoglobin and AE1 occurs in the erythrocyte membrane after treatment of deoxygenated red blood cells with NO solutions (which contain significant-more than 50 μM- contaminating nitrite; Fernandez,*et al.* Inorg Chem 42:2-4, 2003). Further, N_2O_3 generated at the membrane could directly nitrosate the abundant intra-erythrocytic glutathione, eliminating the requirement of transnitrosation reactions with S-nitroso-hemoglobin and thus facilitating rapid export of low molecular weight S-nitrosothiol by simple diffusion across the erythrocyte membrane (Figure 5). A nitrite-hemoglobin chemistry supports a role for the red cell in oxygen-dependent NO homeostasis and provides a mechanism for the observations of multiple research groups that red blood cells and plasma "loaded" with NO, by exposure to NO in high concentration in solution or to NO gas or donors (in equilibria with high concentrations of nitrite), can export NO and induce vasodilation *in vitro* and *in vivo* (Rassaf *et al., J Clin Invest* 109:1241-1248, 2002; Fox-Robichaud *et al., J Glitz Invest* 101:2497-2505, 1998; McMahon *et al., Nat Med* 3:3, 2002; Cannon *et al., J Clin Invest* 108:279-287, 2001; Gladwin *et al., J Biol Chem* 21:21, 2002; Gladwin *et al., Circulation* 107:271-278, 2003; Schechter *et al., N Engl J Med* 348:1483-1485, 2003).

In addition to the reaction of nitrite with deoxyhemoglobin, reactions with deoxy-myoglobin, -cytoglobin and -neuroglobin or with other endothelial cell heme proteins may also be important. Such chemistry would occur between tissue nitrite and deoxy-myoglobin in vascular and skeletal muscle, thus contributing to hypoxic vasodilation and hypoxic potentiation of NO donors. The P_{50} of these globin monomers is approximately 3-5 mm Hg, placing their equilibrium deoxygenation point in the range of tissue pO_2 (0-10 mm Hg) during metabolic stress, such as exercise. Such a low oxygen tension reduces oxygen availability as substrate for NO synthesis, however, the tissue nitrite stores could then be reduced to NO and 5-nitrosothiol, thus sustaining critical vasodilation.

VII. Methods of Use

Therapeutic application of nitrite now can be used to provide selective vasodilation in a subject, and particularly to hypoxemic and ischemic tissue in the subject, and will be useful to treat hemolytic conditions such as sickle cell disease, where free hemoglobin released during hemolysis scavenges NO and disrupts NO-dependent vascular function. Nitrite is expected to not only inhibit the ability of free hemoglobin to scavenge NO by oxidizing it to methemoglobin, but also to generate NO in tissue beds with low oxygen tension. Thus, the applied nitrite will preferentially release nitric oxide at areas of low oxygen tension, thereby providing localized vasodilation and/or increased blood flow.

Nitrites can be administered to a subject to increase blood flow to a tissue of the subject, for example, to increase blood flow to a tissue, for instance a tissue with low oxygen tension; to cause vasodilation; to decrease a subject's blood pressure; to treat a subject having a condition associated with elevated blood pressure; to treat a hemolytic condition; to treat vascular complications associated with treatments or conditions that cause hemolysis; to treat pulmonary hypertension, cerebral vasospasm, or low blood flow to organs (such as ischemia reperfusion injury to organs including brain, heart, kidney, and liver); and/or to treat organs before and after transplantation.

The vasodilator properties of nitrite and the mechanisms for its bioactivation were investigated as described herein. Sodium nitrite infused at 36 μmoles per minute into the forearm brachial artery of 18 normal volunteers resulted in a regional nitrite concentration of 222 μM and, surprisingly, a 175% increase in resting forearm blood flow. Increased blood flow was observed at rest, during NO synthase inhibition and with exercise. The nitrite infusion also surprisingly resulted in increased tissue perfusion, as demonstrated by increases in venous hemoglobin-oxygen saturation, partial pressure of oxygen, and pH. Increased systemic concentrations of nitrite (16 μM) significantly reduced mean arterial blood pressure.

In an additional ten subjects, the dose of nitrite was reduced 2-logs, resulting in a forearm nitrite concentration of 2 μM at rest and 0.9 μM during exercise (Figure 3). These concentrations of nitrite surprisingly significantly increased blood flow at rest and during NO synthase inhibition, with and without exercise.

Nitrite infusions were associated with the rapid formation of erythrocyte iron-nitrosyl-hemoglobin, and to a lesser extent, S-nitroso-hemoglobin across the forearm vasculature. Formation of these NO-Hb adducts was inversely proportional to the oxyhemoglobin saturation. Additionally, vasodilation of rat aortic rings and the formation of both NO gas and NO-modified hemoglobin from the nitrite reductase activity of deoxyhemoglobin and deoxygenated erythrocytes was observed, a result that links tissue hypoxia, hemoglobin allostery, and nitrite bioactivation. These results indicate that physiological levels of blood and tissue nitrite are a major bioavailable pool of NO that contributes to vaso-regulation and provide a mechanism for hypoxic vasodilation via reaction of vascular nitrite with deoxygenated heme proteins in tissue and/or the erythrocyte.

The findings described herein that administration of nitrite reduces blood pressure and increases blood flow are unexpected and surprising because published reports to date teach the person of ordinary skill in the art that pharmacological levels of nitrites (below about 100-200 μM), when administered to subjects, lack intrinsic vasodilatory properties (Lauer *et al.*, *Proc Natl Acad Sci USA*, 98:12814-9, 2001).

It is also believed that pharmaceutically acceptable salts of nitrite can be infused into patients with hemolytic disease, such as sickle cell disease, to improve blood flow, limit ischemia-reperfusion tissue injury, and oxidize cell-free plasma Hb. These effects should be useful in the treatment of sickle cell vaso-occlusive pain crisis, stroke (brain ischemia) and the acute chest syndrome.

VIII. Formulations and Administration

Nitrites, including their salts, are administered to a subject in accordance to methods provided herein, in order to decrease blood pressure and/or increase vasodilation in a subject. Administration of the nitrites in accordance with the present disclosure may be in a single dose, in multiple doses, and/or in a continuous or intermittent manner, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the nitrites may be essentially continuous over a preselected period of time or may be in a series of spaced doses. The amount administered will vary depending on various factors including, but not limited to, the condition to be treated and the weight, physical condition, health, and age of the subject. Such factors can be determined by a clinician employing animal models or other test systems that are available in the art.

To prepare the nitrites, nitrites are synthesized or otherwise obtained and purified as necessary or desired. In some embodiments of the disclosure, the nitrite is a pharmaceutically-acceptable salt of nitrite, for example, sodium nitrite. In some embodiments of the disclosure, the nitrite is not ethyl nitrite. In some embodiments of the disclosure, the sodium nitrite is not on a medical devise, for example, not on a stent. In some embodiments of the disclosure, the nitrite is not in the form of a gel. The nitrites can be adjusted to the appropriate concentration, and optionally combined with other agents. The absolute weight of a given nitrite included in a unit dose can vary. In some embodiments of the disclosure, the nitrite is administered as a salt of an anionic nitrite with a cation, for example, sodium, potassium, or arginine.

One or more suitable unit dosage forms including the nitrite can be administered by a variety of routes including topical, oral (for instance, in an enterically coated formulation), parenteral (including subcutaneous, intravenous, intramuscular and intraperitoneal), rectal, dermal, transdermal, intrathoracic, intrapulmonary and intranasal (respiratory) routes.

The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods known to the pharmaceutical arts. Such methods include the step of mixing the nitrite with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system. By "pharmaceutically acceptable" it is meant a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious or unsuitably harmful to the recipient thereof. The therapeutic compounds may also be formulated for sustained release, for example, using microencapsulation (see WO 94/ 07529, and U.S. Patent No. 4,962,091).

The nitrites may be formulated for parenteral administration (*e.g.*, by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, small volume infusion containers or in multi-dose containers. Preservatives can be

added to help maintain the shelve life of the dosage form. The nitrites and other ingredients may form suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the nitrites and other ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, *e.g.*, sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable carriers and vehicles that are available in the art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol," polyglycols and polyethylene glycols, C_1-C_4 alkyl esters of short-chain acids, ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol," isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

It is possible to add other ingredients such as antioxidants, surfactants, preservatives, film-forming, keratolytic or comedolytic agents, perfumes, flavorings and colorings. Antioxidants such as t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol and its derivatives can be added.

The pharmaceutical formulations of the present disclosure may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are available in the art. Examples of such substances include normal saline solutions such as physiologically buffered saline solutions and water. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present disclosure include water and physiologically acceptable buffered saline solutions, such as phosphate buffered saline solutions. Merely by way of example, the buffered solution can be at a pH of about 6.0-8.5, for instance about 6.5-8.5, about 7-8.

The nitrites can also be administered via the respiratory tract. Thus, the present disclosure also provides aerosol pharmaceutical formulations and dosage forms for use in the methods of the disclosure. In general, such dosage forms include an amount of nitrite effective to treat or prevent the clinical symptoms of a specific condition. Any attenuation, for example a statistically significant attenuation, of one or more symptoms of a condition that has been treated pursuant to the methods of the present disclosure is considered to be a treatment of such condition and is within the scope of the disclosure.

For administration by inhalation, the composition may take the form of a dry powder, for example, a powder mix of the nitrite and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, *e.g.*, gelatin or blister packs from which the powder may be administered with the aid of an inhalator, insufflator, or a metered-dose inhaler (see, for example, the pressurized metered dose inhaler (MDI) and the dry powder inhaler disclosed in Newman, S. P. in *Aerosols and the Lung*, Clarke, S. W. and Davia, D. eds., pp. 197-224, Butterworths, London, England, 1984).

Nitrites may also be administered in an aqueous solution, for example, when administered in an aerosol or inhaled form. Thus, other aerosol pharmaceutical formulations may include, for example, a physiologically acceptable buffered saline solution. Dry aerosol in the form of finely divided solid compound that is not dissolved or suspended in a liquid is also useful in the practice of the present disclosure.

For administration to the respiratory tract, for example, the upper (nasal) or lower respiratory tract, by inhalation, the nitrites can be conveniently delivered from a nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may include a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Nebulizers include, but are not limited to, those described in U.S. Patent Nos. 4,624,251; 3,703,173; 3,561,444; and 4,635,627. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co. (Valencia, CA). For intra-nasal administration, the therapeutic agent may also be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker). The nitrites may also be delivered via an ultrasonic delivery system. In some embodiments of the disclosure, the nitrites may be delivered via an endotracheal tube. In some embodiments of the disclosure, the nitrites may be delivered via a face mask.

The present disclosure further pertains to a packaged pharmaceutical composition such as a kit or other container. The kit or container holds a therapeutically effective amount of a pharmaceutical composition of nitrite and instructions for using the pharmaceutical composition for treating a condition.

IX. Combination Therapies

Furthermore, the nitrite may also be used in combination with one or more other therapeutic agents, for example, pain relievers, anti-inflammatory agents, antihistamines, and the like, whether for the conditions described herein or some other condition. By way of example, the additional agent is one or more selected from penicillin, hydroxyurea, butyrate, clotrimazole, arginine, or a phosphodiesterase inhibitor (such as sildenafil).

It is believed that any therapy used with or suggested for use in combination with NO therapy could be used with the nitrite therapies described herein.

The following example is provided to illustrate certain particular features and/or embodiments. This example should not be construed to limit the invention to the particular features or embodiments described.

Example 1

Nitrite has vasodilatory properties *in vivo*

This example provides a demonstration that nitrite, administered by infusion to the forearm of human subjects, is an effective vasodilator.

Methods

Human subjects protocol.

The protocol was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and informed consent was obtained from all volunteer subjects. Nine men and nine women, with an average age of 33 years (range 21 - 50 years), participated in the study. An additional 10 subjects returned three-six months later for a second series of experiments with low dose nitrite infusion. Volunteers had a normal hemoglobin concentration, and all were in excellent general health without risk factors for endothelial dysfunction (fasting blood sugar >120 mg/dL, low-density lipoprotein cholesterol >130 mg/dL, blood pressure >145/95 mmHg, smoking within two years, cardiovascular disease, peripheral vascular disease, coagulopathy, or any other disease predisposing to vasculitis or Raynaud's phenomenon). Subjects with G6PD deficiency, known cytochrome B5 deficiency or a baseline methemoglobin level > 1% were excluded (no screened subjects met these exclusion criteria). Lactating and pregnant females were excluded (one subject with positive HCG levels was excluded). No volunteer subject was allowed to take any medication (oral contraceptive agents allowed), vitamin supplements, herbal preparations, nutriceuticals or other "alternative therapies" for at least one month prior to study and were not be allowed to take aspirin for one week prior to study.

Forearm blood flow measurements

Brachial artery and antecubital vein catheters were placed into the arm, with the intra-arterial catheter connected to a pressure transducer for blood pressure measurements and an infusion pump delivering normal saline at 1 mL/min. After 20 minutes of rest, baseline arterial and venous blood samples were obtained and forearm blood flow measurements were made by strain gauge venous-occlusion plethysmography, as previously reported (Panza *et al.*, *Circulation, 87*, 1468-74, 1993). A series of 7 blood flow measurements were averaged for each blood flow determination. A series of measurements termed Parts I and II were performed in randomized order to minimize a time effect on the forearm blood flow response during nitrite infusion.

Measurement of blood flow and forearm nitrite extraction during NO blockade and repetitive exercise

Part I: Following 20 minutes of 0.9% NaCl (saline) solution infusion at 1 mL/min into the brachial artery, arterial and venous blood samples were obtained for the assays described below and forearm blood flow measured. Exercise was performed by repetitive hand-grip at one-third of the

predetermined maximum grip strength using a hand-grip dynamometer (Technical Products Co.) (Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 11482-11487, 2000; Cannon *et al.*, *J Clin Invest, 108*, 279-87, 2001). Each contraction lasted for 10 seconds followed by relaxation for 5 seconds. Following 5 minutes of exercise, forearm blood flow measurements were obtained during relaxation phases of exercise, and arterial and venous samples collected. Following a 20-minute rest period with continued infusion of saline into the brachial artery, repeated baseline blood samples and forearm blood flow measurements were obtained. L-NMMA was then infused at a rate of 1 mL/min (8 μmol/min) into the brachial artery. Following 5 minutes of L-NMMA infusion, forearm blood flow was measured, and arterial and venous blood samples obtained. Forearm exercise was then initiated in that arm during continued L-NMMA infusion. Forearm blood flow was measured and blood samples obtained after 5 minutes of exercise during continued L-NMMA infusion (Figure 1).

Part II: After a 30 minute rest period with continued infusion of saline, baseline measurements were obtained, the saline infusion was then stopped, and infusion of nitrite ($NaNO_2$ 36 μmol/ml in 0.9% saline) at 1 ml/min was started. Sodium nitrite for use in humans was obtained from Hope Pharmaceuticals (300 mg in 10 ml water) and 286 mg was diluted in 100 ml 0.9% saline by the Pharmaceutical Development Service to a final concentration of 36 μmol/ml. For the final 9 subjects studied, 0.01-0.03 mM sodium bicarbonate was added to the normal saline, so as to titrate pH to 7.0-7.4. The nitrite solution was light protected and nitrite levels and free NO gas in solution measured by reductive chemiluminescence after all experiments (Gladwin *et al.*, *J Biol Chem, 21*, 21, 2002). Only 50.5 ± 40.5 nM NO was present in nitrite solutions and was unaffected by bicarbonate buffering. There was no correlation between NO levels in nitrite solutions and blood flow effects of nitrite ($r = -0.23$; $P=0.55$). After 5 minutes of nitrite infusion, forearm blood flow measurements and blood samples were obtained, with brief interruption of the nitrite infusion to obtain the arterial sample. With continued nitrite infusion, exercise was performed as described previously, with forearm blood flow measurements and blood samples obtained as described above. The nitrite infusion was stopped and saline infusion re-started during the subsequent 30-minute rest period. Following second baseline measurements, the nitrite infusion was re-initiated, along with L-NMMA at 8 μmol/min. Five minutes later, forearm blood flow measurements were performed and blood samples obtained followed by 5 minutes of exercise with continuation of nitrite and L-NMMA infusions. Final forearm blood flow measurements and blood samples obtained. At all time points during part II, blood samples were obtained from the contralateral arm antecubital vein for determination of methemoglobin and systemic levels of NO-modified hemoglobin (Figure 2, 3, and 4). The total dose of sodium nitrite infused was 36 μmol/min x 15 minutes x 2 infusions = 1.08 mmol = 75 mg (MW $NaNO_2$ = 69).

In additional studies in 10 subjects the same stages of Parts I and II protocol were followed with infusion of low dose nitrite ($NaNO_2$ 0.36 μmol/ml in 0.9% saline, infused at 1 ml/min).

Arterial and venous pH, pO_2, and pCO_2, were measured at the bedside using the i-STAT system (i-STAT Corporation, East Windsor, NJ) and methemoglobin concentration and hemoglobin oxygen saturation measured by co-oximetry.

Measurement of red blood cell S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin.

S-nitroso-hemoglobin is unstable in the reductive red blood cell environment and rapidly decays in a temperature and redox dependent fashion, independent of oxygen tension (Gladwin *et al.*, *J Biol Chem*, 21:21, 2002). To stabilize the S-nitroso-hemoglobin for measurement, the red blood cell must be rapidly oxidized with ferricyanide. Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" of PBS containing 1% NP-40 (to solubilize membranes), 8 mM NEM (to bind free thiol and prevent artefactual S-nitrosation), 0.1 mM DTPA (to chelate trace copper), and 4 mM ferricyanide and cyanide (to stabilize S-nitrosohemoglobin and prevent artefactual ex-vivo iron-nitrosylation during processing). The samples were desalted across a 9.5 mL bed volume Sephadex G25 column to eliminate nitrite and excess reagents and partially purify hemoglobin (99% hemoglobin preparation). The hemoglobin fraction was quantified by the method of Drabkin, and hemoglobin fractions reacted with and without mercuric chloride (1:5 $HgCl_2$:heme ratio- used to differentiate S-nitrosothiol which is mercury labile versus iron-nitrosyl which is mercury stable) and then in 0.1 M HCL/0.5%sulfanilamide (to eliminate residual nitrite; Marley *et al.*, *Free Radic Res, 32*, 1-9, 2000). The samples were then injected into a solution of tri-iodide (I_3^-) in-line with a chemiluminescent nitric oxide analyzer (Sievers, Model 280 NO analyzer, Boulder, CO). The mercury stable peak represents iron-nitrosyl-hemoglobin. This assay is sensitive and specific for both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin to 5 nM in whole blood (0.00005% S-NO per heme) (Gladwin *et al.*, *J Biol Chem, 21*, 21, 2002).

Analysis was initially performed using red blood cell pellet, however, despite placing the sample in ice and immediately separating plasma from erythrocyte pellet, NO formed in the venous blood *ex vivo*. To measure the true *in vivo* levels, whole blood was mixed at the bedside 1:10 in the "NO-hemoglobin stabilization solution". Plasma S-nitroso-albumin formation was negligible during nitrite infusion so this bedside whole blood assay was used to limit processing time and thus more accurately characterize the *in vivo* chemistry. In a series of validation experiments, both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were stable in the "NO-hemoglobin stabilization solution" for 20 minutes at room temperature with no artifactual formation or decay of NO-modified species (n=6).

Chemiluminescent detection of NO gas released from deoxyhemoglobin and deoxygenated erythrocytes following nitrite addition.

To determine whether free NO radical can form from the reaction of nitrite and deoxyhemoglobin, 100 and 200 μM nitrite was mixed with 5 mL of 660 and 1000 μM deoxygenated erythrocytes in a light protected reaction vessel purged with helium or oxygen (both 21% and 100%) in-line with a chemiluminescent NO analyzer (Seivers, Boulder, CO). After allowing equilibration for 5 minutes, nitrite was injected and the rate of NO production measured. Nitrite was injected into PBS as a control and into 100 μM hemoglobin to control for the hemolysis in the 660 and 1000 μM deoxygenated erythrocyte solutions. At the end of all experiments the visible absorption spectra of the supernatant and erythrocyte reaction mixture was analyzed and hemoglobin composition deconvoluted using a least-squares algorithm. There was less than 100 μM hemolysis in the system, no hemoglobin denaturation, and significant formation of iron-nitrosyl-hemoglobin. The NO production from erythrocyte suspensions exceeded that produced from the hemolysate control, consistent with NO export from the erythrocyte.

Statistical analysis.

An *a priori* sample size calculation determined that 18 subjects would be necessary for the study to detect a 25% improvement in forearm blood flow during nitrite infusion when forearm NO synthesis had been inhibited by L-NMMA compared with normal saline infusion control values (alpha=0.05, power=0.80). Two-sided P values were calculated by paired t-test for the pair-wise comparisons between baseline and L-NMMA infusion values, between baseline and exercise values, and between nitrite and saline control values at comparable time-points of the study. Repeated measures ANOVA were performed for artery-to-vein gradients of NO species during basal, L-NMMA infusion, and exercise conditions. Measurements shown are mean ± SEM.

Results and Discussion

Eighteen healthy subjects (9 males, 9 females; age range 21 to 50 years) were enrolled in a physiological study to determine if nitrite is a vasodilator and to examine nitrite's *in vivo* chemistry. Part I of the protocol was designed to measure the normal hemodynamic and metabolic responses to exercise and to inhibition of NO synthesis within the forearm as a control for Part II of the protocol, in which these interventions were performed during nitrite infusion. Initial baseline measurements included a mean blood pressure of 85.6 ± 3.7 mm Hg and forearm blood flow of 4.0 ± 0.3 ml/min per 100 mL tissue (Figure 1A). Repetitive hand-grip forearm exercise increased blood flow approximately 600% over resting values, and significantly decreased ipsilateral venous hemoglobin oxygen saturation, pO_2, and pH, consistent with increased oxygen consumption and CO_2 generation. Following a 20-minute rest period, repeat hemodynamic measurements showed an approximate 10% higher forearm blood flow, but no change in systemic blood pressure or forearm venous hemoglobin oxygen saturation, pO_2 and pH values compared with the initial baseline values (Figure 1B). The NO

synthase inhibitor L-NMMA was then infused into the brachial artery at 8 μmol/min for 5 minutes, significantly reducing forearm blood flow by approximately 30% and significantly reducing venous hemoglobin oxygen saturation, pO_2 and pH values. Repeated forearm exercise during continued L-NMMA infusion increased blood flow, but to a significantly lower peak value compared with exercise alone ($P<0.001$). In addition, hemoglobin oxygen saturation, pO_2 and pH were significantly lower during exercise with L-NMMA than with exercise without regional NO synthase inhibition ($P<0.001$, $P<0.005$ and $P=0.027$, respectively). Mean arterial blood pressure was unchanged during all components of Part I of the protocol.

Figure 1 depicts hemodynamic and metabolic measurements at baseline and during exercise, without (Figure 1A) and with (Figure 1B) inhibition of NO synthesis in 18 subjects. Mean arterial pressure (MAP), forearm blood flow (FBF), and venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2), and pH are shown for all experimental conditions. These interventions and measurements (part I of the protocol) served as a control for Part II of the protocol, in which these interventions were performed during nitrite infusion.

To determine whether nitrite has vasoactivity in humans, in Part II of the protocol sodium nitrite in bicarbonate-buffered normal saline (final concentration 36 μmol/ml) was infused into the brachial arteries of these 18 subjects to achieve an estimated intravascular concentration of approximately 200 μM (Lauer *et al.*, *Proc Natl Acad Sci U S A, 98*, 12814-9, 2001). Following repeat baseline measurements and infusion of sodium nitrite at 1 mL/min for 5 minutes, nitrite levels in the ipsilateral antecubital vein increased from 3.32 ± 0.32 to 221.82 ± 57.59 μM (Figure 2A). Forearm blood flow increased 175% over resting values; venous hemoglobin oxygen saturation, pO_2 and pH levels significantly increased over pre-infusion values, consistent with increased perfusion of the forearm.

Systemic levels of nitrite were 16 μM as measured in the contralateral arm and were associated with a systemic effect of decreased mean blood pressure of approximately 7 mm Hg. Consistent with immediate NO generation from nitrite during an arterial-to-venous transit, iron-nitrosylated-hemoglobin in the ipsilateral antecubital vein increased from 55.7 ± 11.4 to 693.4 ± 216.9 nM during the nitrite infusion. During forearm exercise with continuation of the nitrite infusion, blood flow increased further, with evidence of metabolic stress by virtue of reduction in forearm venous hemoglobin oxygen saturation, pO_2 and pH levels from baseline values. Venous nitrite levels declined, consistent with increased blood flow to the forearm diluting the concentration of infused nitrite. Despite decreasing forearm nitrite concentrations during exercise, iron-nitrosyl-hemoglobin levels increased (Figure 2A).

Following cessation of nitrite infusion and substitution of saline as the intra-arterial infusate for 30 minutes, repeat baseline measurements showed persistent elevations in systemic levels of nitrite, iron-nitrosyl-hemoglobin and methemoglobin (Figure 2B) over values obtained prior to the infusion of nitrite almost one hour before. In addition, persistence of a vasodilator effect was also apparent, as forearm blood flow was significantly higher (4.79 ± 0.37 versus 3.94 ± 0.38 mL/min per

100 mL tissue, P=0.003) and systemic blood pressure significantly lower (82.1 ± 3.7 versus 89.2 ± 3.5 mm Hg, P=0.002) than initial pre-nitrite infusion values. During re-infusion into the brachial artery of sodium nitrite 36 μmol/ml, combined with L-NMMA 8 μmol/min in order to again inhibit regional synthesis of NO, similar vasodilator effects of nitrite on resting and exercise forearm blood flow were seen as during nitrite infusion without L-NMMA (Figure 2B). This stands in contrast to the vasoconstrictor effect of NO synthase inhibition with L-NMMA observed in Part I of the protocol (Figure 1B). Venous nitrite and iron-nitrosyl-hemoglobin levels followed similar patterns during NO inhibition as during the initial nitrite infusion.

Figure 2 depicts the effects of infusion of sodium nitrite ($NaNO_2$) in bicarbonate-buffered normal saline (0.9%; final concentration 36 μmol/ml) into the brachial arteries of 18 healthy subjects at 1 ml/min for 5 minutes at baseline and continued during exercise. Figure 2A depicts the effects without inhibition of NO synthesis. Figure 2B depicts the effects with inhibition of NO synthesis. Values for mean arterial blood pressure (MAP), forearm blood flow (FBF), venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2) and pH, venous nitrite, venous iron-nitrosyl-hemoglobin and venous methemoglobin are shown for all experimental interventions.

As a test of the physiological relevance of vascular nitrite as a vasodilator, nitrite concentrations were decreased by 2-logs to 400 nmol/mL. An infusion of 1 mL/min for five minutes in 10 subjects significantly increased forearm blood flow in all ten subjects from 3.49 ± 0.24 to 4.51 ± 0.33 ml/min per 100 mL tissue (Figure 3A; P=0.0006). Blood flow significantly increased at rest and during NO synthase inhibition with and without exercise (Figure 3B; P<0.05 during all conditions). Mean venous nitrite levels increased from 176 ± 17 nM to 2564 ± 462 nM following a five-minute infusion and exercise venous nitrite levels decreased to 909 ± 113 nM (secondary to dilutional effects of increased flow during exercise; Figure 3C). Again, the vasodilator effects of nitrite were paralleled with an observed formation of both iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin across the forearm circulation (Figure 3D; described below). These data indicate that basal levels of nitrite, from 150-1000 nM in plasma to 10,000 nM in vascular tissue, contribute to resting vascular tone and hypoxic vasodilation.

Figure 3 depicts the effects of infusion of low-dose sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 10 healthy subjects at baseline and during exercise, without and with inhibition of NO synthesis. Figure 3A depicts forearm blood flow at baseline and following a five-minute in fusion of $NaNO_2$ (0.36 μmol/ml in 0.9% saline, infused at 1 ml/min). Figure 3B depicts forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion with and without exercise stress. Figure 3C depicts venous levels of nitrite from the forearm circulation at the time of blood flow measurements. Figure 3D depicts venous levels of S-nitroso-hemoglobin (S-NO) and iron-nitrosyl-hemoglobin (Hb-NO) at baseline and following nitrite infusion during exercise stress.

The vasodilatory property of nitrite during basal blood flow conditions, when tissue pO_2 and pH are not exceedingly low, was unexpected. These results indicate that the previously hypothesized

mechanisms for nitrite reduction, nitrite disproportionation and xanthine oxidoreductase activity, both of which require extremely low pO_2 and pH values not typically encountered in normal physiology, are complemented *in vivo* by additional factors that serve to catalyze nitrite reduction. While ascorbic acid and other reductants, present in abundance in blood, can provide necessary electrons for nitrous acid reduction, such that the reaction might occur at physiologically attainable pH levels, it is herein reported that deoxyhemoglobin effectively reduces nitrite to NO, within one half-circulatory time. This mechanism provides a graded production of NO along the physiological oxygen gradient, tightly regulated by hemoglobin oxygen desaturation.

Intravascular formation of NO and S-nitrosothiol by reaction of nitrite with intraerythrocytic deoxyhemoglobin

Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" and the iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin content determined by tri-iodide-based reductive chemiluminescence and electron paramagnetic resonance spectroscopy as described in Methods. The baseline levels of S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were at the limits of detection (<50 nM or 0.0005% NO per heme) with no artery-to-vein gradients. Following nitrite infusion in Part II of the protocol venous levels of both iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin rose strikingly (Figure 4A). The formation of both NO-hemoglobin adducts occurred across the vascular bed, a half-circulatory time of less than 10 seconds. The rate of NO formation, measured as iron-nitrosyl and S-nitroso-hemoglobin and quantified by subtraction of the arterial from the venous levels with the difference being multiplied by blood flow, increased greatly during exercise, despite a significant decrease in the venous concentration of nitrite secondary to increasing blood flow diluting the regional nitrite concentration (Figure 4A; $P=0.006$ for iron-nitrosyl-hemoglobin and $P=0.02$ for S-nitroso-hemoglobin by repeated measures ANOVA).

Figure 4A depicts formation of iron-nitrosyl-hemoglobin (black squares) and S-nitroso-hemoglobin (red circles) during nitrite infusion at baseline, during nitrite infusion and during nitrite infusion with exercise, quantified by subtraction of the arterial from the venous levels and multiplying the result by blood flow. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation in the human circulation during nitrite infusion (for iron-nitrosyl-hemoglobin $r=-0.7$, $p<0.0001$, for S-nitroso-hemoglobin $r=-0.45$, $p=0.04$) (Figure 4B). Hemoglobin oxygen saturation was measured from the antecubital vein by co-oximetry. Asterix in all figures signify $P<0.05$ by paired t test or repeated measures analysis of variance.

To determine whether free NO radical can form from the reaction of nitrite and deoxyhemoglobin, 100 and 200 μM nitrite was reacted with deoxygenated erythrocytes (5 mL volume containing a total of 660 and 1000 μM in heme) in a light protected reaction vessel purged with helium in-line with a chemiluminescent NO analyzer (Seivers, Boulder, CO). As shown in

Figure 5A and 5B, the injection of nitrite into a solution of deoxygenated erythrocytes resulted in the liberation of NO into the gas phase. There was no release from nitrite in buffer control under the same conditions, and significantly less NO was released upon nitrite addition to oxygenated erythrocytes (21% and 100% oxygen). The observed rate (determined by the assessment of the area under the curve of increased steady-state NO generation following nitrite injection calculated over 120 seconds) of NO production in the 5 mL reaction volume was consistent with 47 pM NO production per second (corresponding to an estimated 300 to 500 pM NO production per second in whole blood). While NO formation rates in this experimental system may not be extrapolated to rates of NO formation *in vivo*, the experiments are consistent with two important concepts: 1) A fraction of free NO can escape auto-capture by the remaining heme groups; this is likely only possible because nitrite is only converted to NO by reaction with deoxyhemoglobin and its "leaving group" is the met(ferric)heme protein which will limit scavenging and inactivation of NO (Doyle *et al.*, *J Biol Chem, 256*, 12393-12398, 1981); and 2) The rate of NO production is increased under anaerobic conditions, consistent with a nitrite-deoxyhemoglobin reaction.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments, and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.